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13. ABSTRACT ***(Maximum 200 Words)***

The **purpose** of this proposal is to evaluate the ability of a replication-restrictive adenovirus (Ad-OC-E1a) to specifically target and lyse cells of an androgen independent prostate cancer osseous metastasis, which account for a majority of the morbidity and mortality experience by men with prostate cancer. The **scope** of this project to perform the studies outlined in proposal to prove the hypothesis that conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. More specifically, Specific Aim I seeks to evaluate the specificity of the tumor-restrictive replication of Ad-OC-E1a using in vitro assays on prostate and non-prostate cancer cells. The ability of the Ad-OC-E1a to have at least a 100 fold killing differential favoring OC + cell lines (LNCaP, C4-2, PC-3) over OC- cell lines (LOVO, PrSC). Specific Aim II evaluates the growth inhibition of human prostate cancer xenografts attributable to Ad-OC-E1a administration, as well as, the tissue distribution and toxicity profile of such injections. The ability to Ad-OC-E1a to completely destroy both androgen-dependent and androgen-independent human prostate cancer cells in xenografts supports the hypothesis being studied.

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Introduction to Revised 2nd Annual Report (revised areas in italics)

The **subject** of this proposal is to develop and test the ability to a genetically modified common cold virus to destroy androgen-independent prostate cancer cells. Androgen-independent prostate cancer cells account for 100% of the mortality associated with prostate cancer. The **purpose** of this proposal is to evaluate the ability of replication-restrictive adenovirus which specifically targets and lyses cells of an androgen independent prostate cancer osseous metastasis. The **scope** of this project to perform the studies outline in the two specific aims to prove the hypothesis that conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. More specifically, Specific Aim I is designed to evaluate the specificity of the tumor-restrictive replication of Ad-OC-E1a using in vitro assays on prostate and non-prostate cancer cells. *Based on the reviewers comments from the first annual review suggested that prostate specific antigen (PSA) expression by prostate cancer cell lines may be down regulated by adenoviral infection a series of experiments were completed to address this question in vitro.* Specific Aim 2 evaluates the growth inhibition of human prostate cancer xenografts attributable to Ad-OC-E1a administration, as well as, the tissue distribution and toxicity profile of such injections. *The request for a revised 2nd Annual Report does not request a revised State of Work but have provided results of additional experiments supported under this grant. Since the 1st Annual Report Review raised several format and editorial issues, I have expanded on the first annual report including the entire report with corrected grammatical text.*

Body

Metastatic prostate cancer remains a daily challenge for the urologist, oncologist and radiation oncologist. The relatively unique pathophysiology underlying the formation of osteoblastic lesions predominately isolated to the bone of men with metastatic prostate cancer has allowed us to transcriptionally target these cancer cells with an osteoblastic promoter, osteocalcin. We have previously utilized a replication-defective adenovirus containing the osteocalcin promoter driving toxic gene expression to target osseous metastases in pre-clinical models[1] and a phase I clinical trial (Ad-OC-TK)[2]. The transcription regulation of transgene expression using tumor- and tissue-specific promoters within adenoviral vectors has been shown to impart tumor or tissue specificity. The osteocalcin promoter has been demonstrated to effectively and safely target prostate cancer[1] and osteosarcoma[3-5] based on the shared osteoblastic phenotype, using a suicide gene therapy approach in preclinical and phase I testing[2].

The lytic replicative cycle of the adenovirus was initially used shortly after the discovery of the adenovirus for the treatment of cancer[6]. The greater understanding of the adenoviral genetic make-up and function has led to the ability to construct conditionally replicating adenoviruses. Restrictive adenoviral replication has been used previously to target p53 mutated[7] cells and more recently PSA producing cells[8]. In this report we demonstrate that previously defined transcriptional specificity of the osteocalcin promoter can be used to destroy prostrate cancer cells by harnessing this adenoviral lytic replication cycle both in vitro and in vivo using relevant models of human hormone-refractory prostate cancer. This is achieved by placing the E1a gene under the transcriptional regulation of the osteocalcin promoter. By constructing an adenoviral vector that has the E1a gene under the control of the murine osteocalcin promoter the osseous metastases, which account for most of the morbidity and eventual mortality attributable to prostate cancer, can be effectively targeted.

Illustration 1 demonstrates the rational underlying this proposal. The osteocalcin promoter has the ability to transcriptionally regulate the production of the E1a protein in osteocalcin positive cell types. The production of this essential protein then directs adenoviral replication and eventual cell lysis. The lytic life cycle of the adenovirus is then allowed to proprogate throughout a tumor mass. The propagation wave will potentially continue until normal osteocalcin negative cells are encountered at the periphery of the tumor.

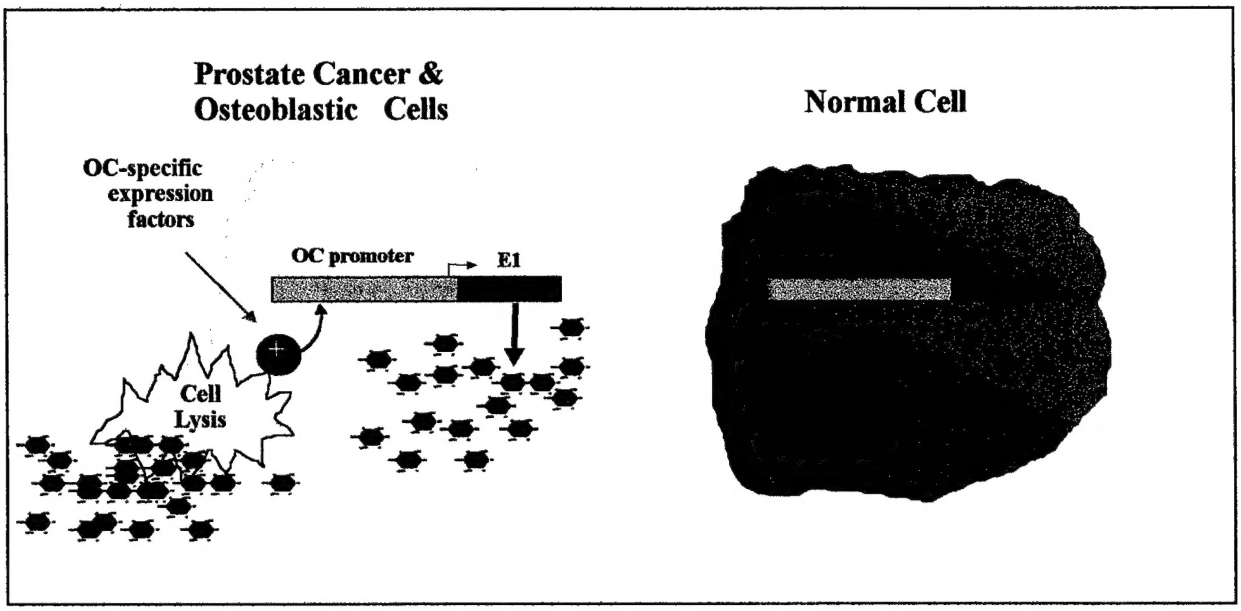


Illustration 1: The Rationale of Osteocalcin-restricted Adenoviral Oncolysis

The completion of the tasks outlined by the Statement of Work of the initial proposal is being performed with slight modifications of the timing as described below. In general, some of the In vivo studies projected for the second year of the proposal were initiated at the 6-month mark and results will be presented. This was a result of equipment difficulties that have been resolved that delayed the initiation of 3-dimensional studies. The research findings will be broken down by Task # as per the "Format Requirements for Preparing Reports".

Task 1 was to amplify, purify, quantify titer and confirm the activity of sufficient viral stocks of Ad-OC-E1a and Ad-CMV-Bgal. This task is ongoing and the PI and other members of the research team continue to improve on the technique in several ways. The PI and others have developed a novel production technique that allows for adenoviral production using a serum-free hollow fiber system[9]. Currently, sufficient Ad-OC-E1a and Ad-CMV-Bgal has been produced to perform the next 6 months of experiments. The adenoviral production continues on schedule. One anticipated problem, which can occur during the amplification of any recombinant adenovirus, was the regeneration of wild-type Ad5, which occurred during the amplification process of Ad-CMV-B-Gal. This contaminant required re-isolation of the virus and subsequent re-amplification of the Ad-CMV-B-Gal virus.

Virus construction and production

The shuttle plasmid pOCE1a was constructed by starting with the shuttle vector pΔE1sp1B, provided by Dr. Frank Graham (McMasters University, Hamilton, Ontario, Canada). pΔE1sp1B contains the right end of the adenovirus type 5 genome, nucleotides 28 to 347, encoding several minor E1a promoters. To stop the transcription initiated from these minor E1a promoters, an SV40 polyadenylation signal (170 bp, Cla I – Hind III fragment, from pXCMVPA, obtained from Dr. Wei-Wei Chang) was cloned into pΔE1sp1B to generate pΔBE1sp1BPA. The Ad5 E1

region, from pX548c (also provided by Frank Graham), was cloned into pΔBE1sp1BPA. These subcloning procedures created the shutter vector pΔE1, which contains the 5'-end of the adenovirus type 5 genome, from nucleotides 28 to 347 and 549 to 5852, with multiple cloning sites between sequences 347 to 549. pΔE1 contains the majority of the E1 region except part of the E1a promoter, nucleotides 348 to 548. A mouse osteocalcin promoter (1370 bp, Not I-EcoR I fragment) from pII1.5, including TATA box, was cloned between the SV40 polyadenlyation site and E1a sequence of pΔE1 to generate **pΔOCE1a**, which has E1a under the transcriptional regulation of the osteocalcin promoter. [pII1.5 was provided by Dr. Gerard Karsenty of the University of Texas M. D. Anderson Cancer Center, Houston Texas.] The sequence of pΔOCE1a was generated by known sequence information and analyzed by restriction enzyme digestion. The virus was generated using traditional method [10] and amplified using both the traditional amplification on monolayers of 293 cells and a hollow fiber production method developed by the investigator[9].

Task 2 was to perform DNA quantification and time course experiments with Ad-OC-E1a using dot blot experiments. The emphasis of this proposal has been shifted to the in vivo studies to prepare for a clinical trial of this virus. This task remains ongoing and results will be presented in the final report.

Task 3 and 4 was to perform in vitro killing assays in a variety of human cell lines. This task has been accomplished and the material, methods, results and discussion follow:

Methods and Materials

Cell lines and cell cultures:

The LNCaP cell line was kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). C4-2 was established from LNCaP tumors propagated in castrated hosts[11; 12]. PC-3 [13]was obtained from the American Type Culture Collection (Rockville, MD). ROS 17/2.8 (ROS), a rat osteoblastic osteosarcoma cell line was generously supplied by Dr. Cindy Farrach-Carson (The University of Texas Dental Branch, Houston, TX). Prostate stromal cells (PrSC) were obtained from Clonetics (Walkersville, MD). LNCaP, C4-2, and PC-3 cell lines were maintained in T-medium [80% Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), 20% F12K (Irving Scientific, Santa Ana, CA), 3 gm/L $NaHCO_3$, 100 units/mL penicillin G, 100μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml transferrin, 0.25 μg/ml biotin, and 25 μg/ml adenine] with 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). ROS cells were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with penicillin (100 units/mL), streptomycin (100mg/ml), and 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). PrSC cells were maintained in Stromal cell basal media, supplemented with the Stromal cell growth media BulletKit (Clonetics, Walkersville, MD). All cell cultures were maintained at 37° C in a humidified atmosphere of 95% air and 5% carbon dioxide. The cells were fed three times per week with fresh growth media.

MTT proliferation assay:

Cells were plated in 24 well plates at the following initial seeding densities in cells/well based on growth rates and prior experience in other in vitro assay on these cells: ROS (10,000), PC-3 (10,000) LNCaP (15,000), PrSC (15,000), C4-2 (40,000). Twenty-four hours after seeding, fresh media was placed on the cells and the cells were exposed to variable concentrations of the Ad-OC-E1a vector (0.01, 0.1, 1, 10, and 100 viral particles/cell; 4 wells each dilution) dissolved in PBS. An additional 4 wells that were treated with vector-free PBS served as controls. The media on all wells was changed every two days. Relative cell numbers were determined at intervals were by incubating the cells with MTT (thiazolyl blue). Briefly, cells were then solubilized in a solution of 10% sodium dodecyl sulfate and 0.1 N hydrochloride for 16 hours. Absorbance was measured at wavelength 550 nm as per the manufactures protocol. The in vitro cell-killing activity of Ad-OC-E1a ranged from 0.01 to100 viral particles per cell was evaluated

on an androgen-independent and metastatic human prostate cancer C4-2 cell line from 0 to 7 days.

Task 3 and 4 Results and Discussion:

The viral lytic effect of Ad-OC-E1a is demonstrated in LNCaP (OC-positive) cells at five day after infection with 1 vp/per cell. The left panel of Figure 1 demonstrates LNCaP cells five days after 1 vp/cell of Ad-CMV-B-Gal exposure. The right panel of Figure 1 demonstrates the significant lytic ability of Ad-OC-E1 on LNCaP five days after 1 vp/cell exposure. The typical cytopathic effect (CPE) is seen in the right panel while absent in the left panel.

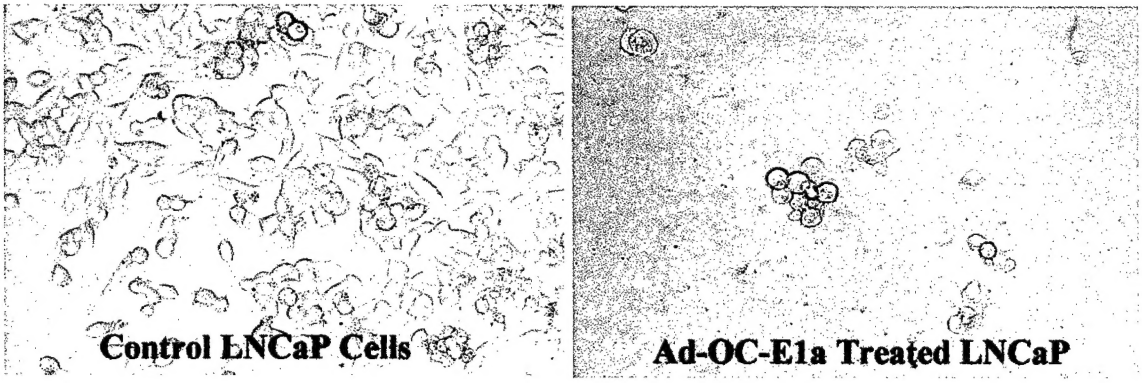


Figure 1. CPE 5 days after Exposure of LNCaP to Ad-OC-E1a (1 vp/cell)

The osteocalcin positive cell lines LNCaP, C4-2 and PC-3 all demonstrate a dose-dependent cell lysis as evaluated by MTT assay. The PrSC serve as an osteocalcin-negative relevant human cell line and demonstrates no cell lysis at day 7. The ROS cell line, expresses higher levels of osteocalcin, but serves a negative control for viral replication since the human adenovirus cannot replicate in rodent cells. **(Figure 2 below)**

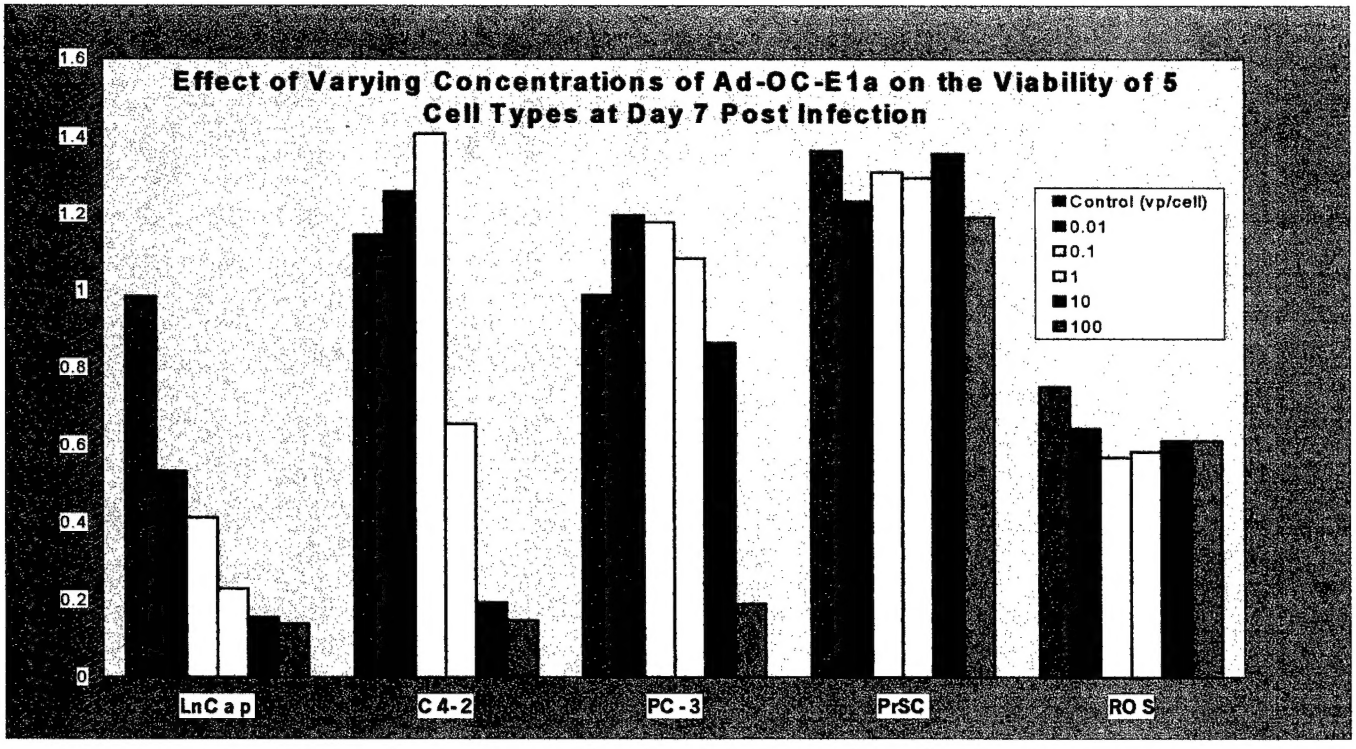


Figure 2: Osteocalin-Resistricted and Viral Particle-Dependent Cell Lysis by MTT Assay

Figures 3 and 4 demonstrate a time course of the Ad-OC-E1a dependent lysis in osteocalcin positive C4-2 cells and osteocalcin negative LOVO cells, respectively. A 100 fold differential is seen between the OC+ C4-2 cell and the OC- LOVO cells. Quantitative PCR will be performed on the DNA extracts from Days 0, 1, 3, 5, 7 to generate the viral production levels and time course.

Figure 3: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in C4-2 Cells as Measured by MTT Assay

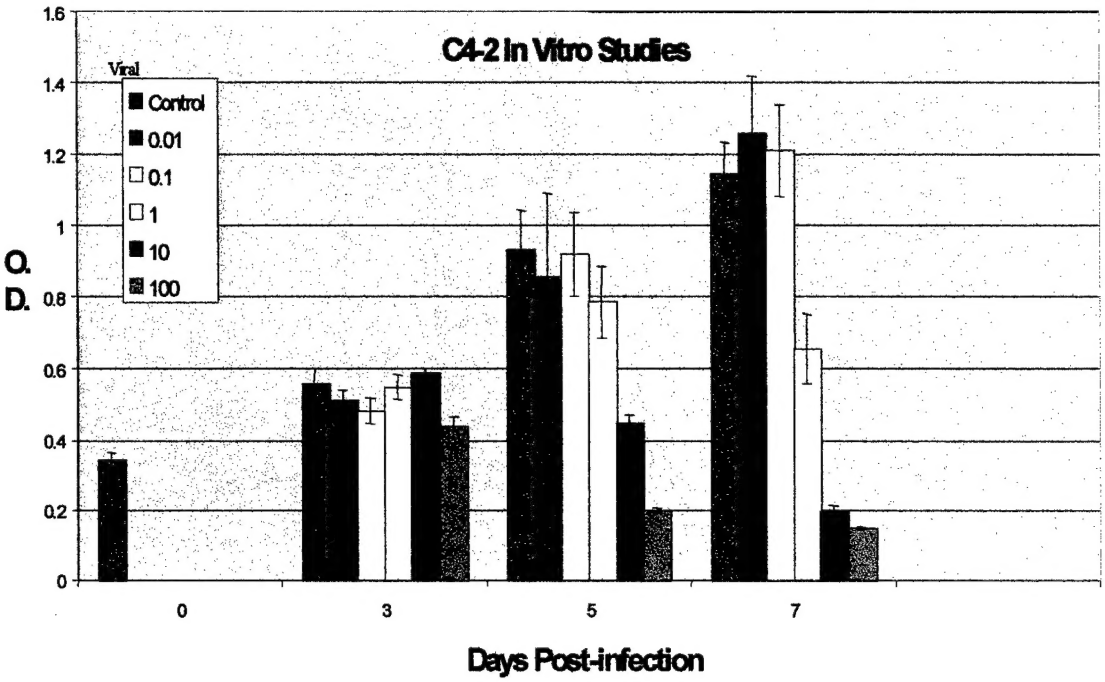


Figure 4: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in LOVO Cells as Measured by MTT Assay

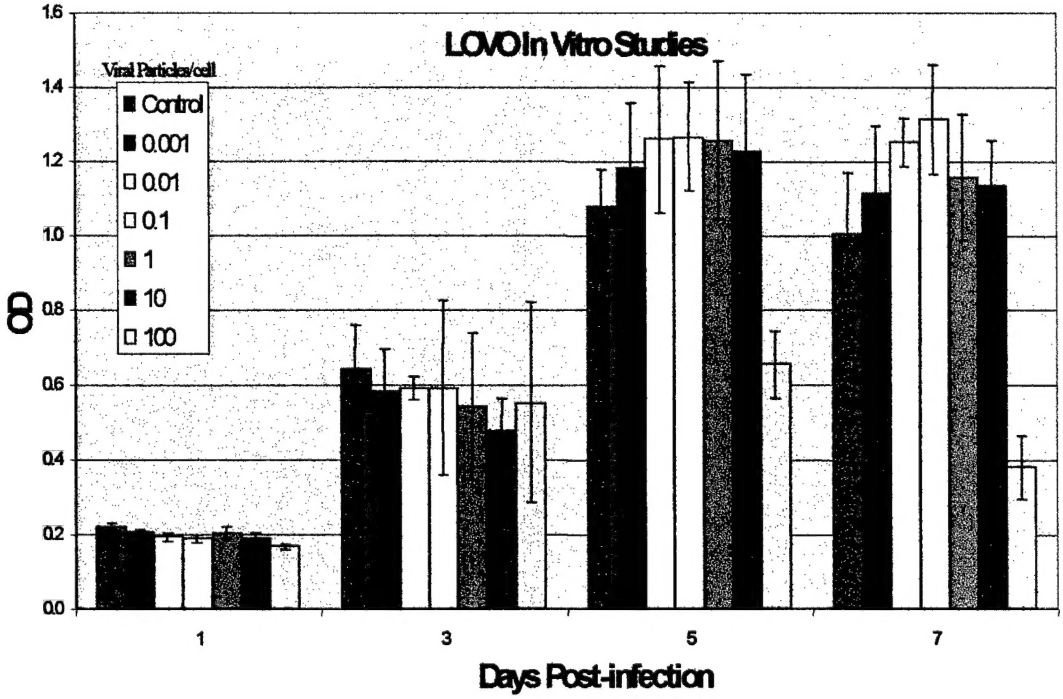


***Figure 5 and 6** would suggest that PSA production by both PSA producing human cell lines LNCaP and C4-2 is not down regulated by adenoviral infection and again confirms the lytic potential of Ad-OC-E1a. Figure 5 illustrates the mean PSA readings in ng/dl and cell counts per 1000 of 4 replicates of LNCaP and C4-2 human prostate cancer cell lines at day 0, 2, 4, 6, 8. LNCaP cells were culture for six days post infection while C4-2 cells were assayed for 8 days post exposure. Four experimental groups were studied. The Control Group was exposed to only normal media, the Control Ly were similar but were exposed to lysis buffer on designated Day, Ad-CMV-GFP Group were exposed to 10 vp/cell on Day O and AD-OC-E1a Group were exposed to 10 vp per cell with media and cells being collected on Day 0, 2, 4, 6, 8 as designated on the x axis. Examination of the cell counts on day 6 for LNCaP and Day 8 for C4-2 again confirms the specific lytic ability of the Ad-OC-E1a virus has on prostate cancer cells.*

Figure 5 PSA Values and Cell Counts

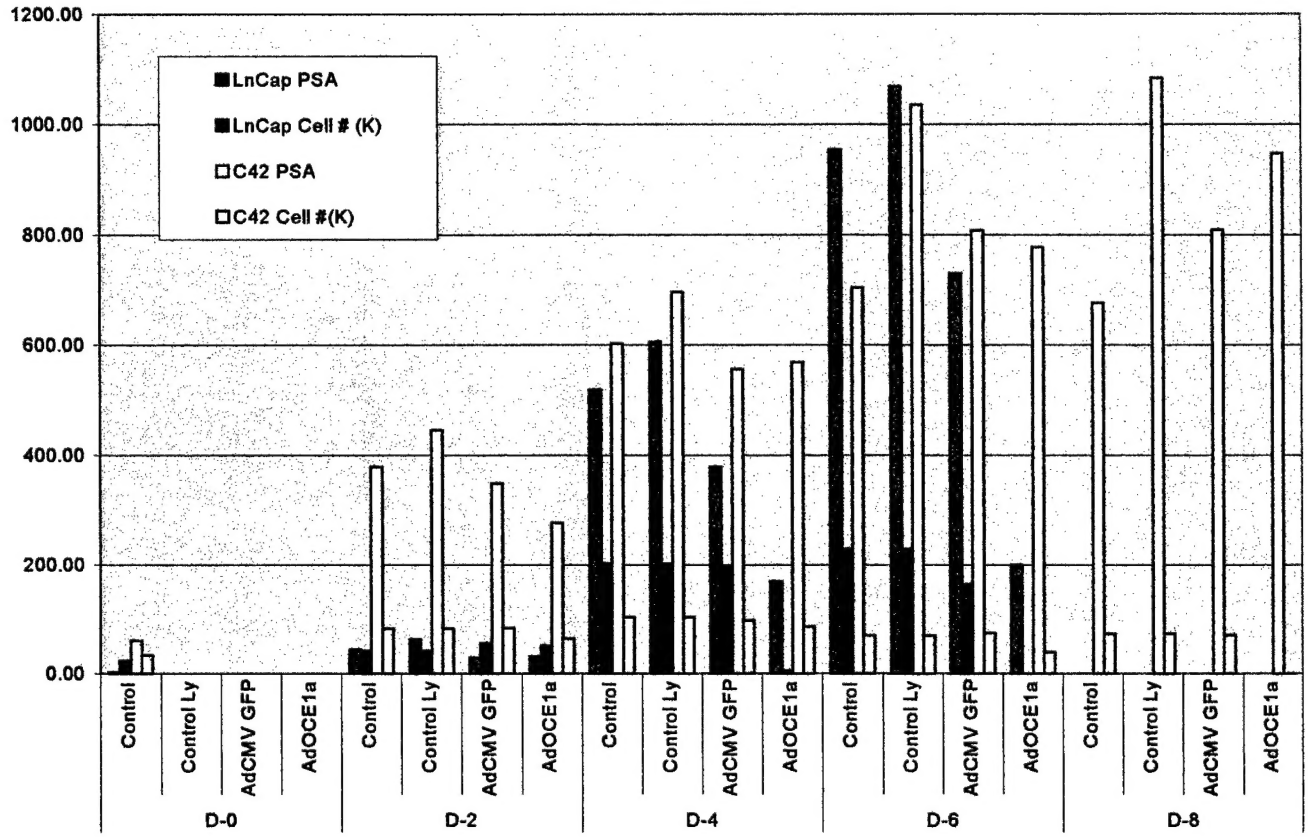
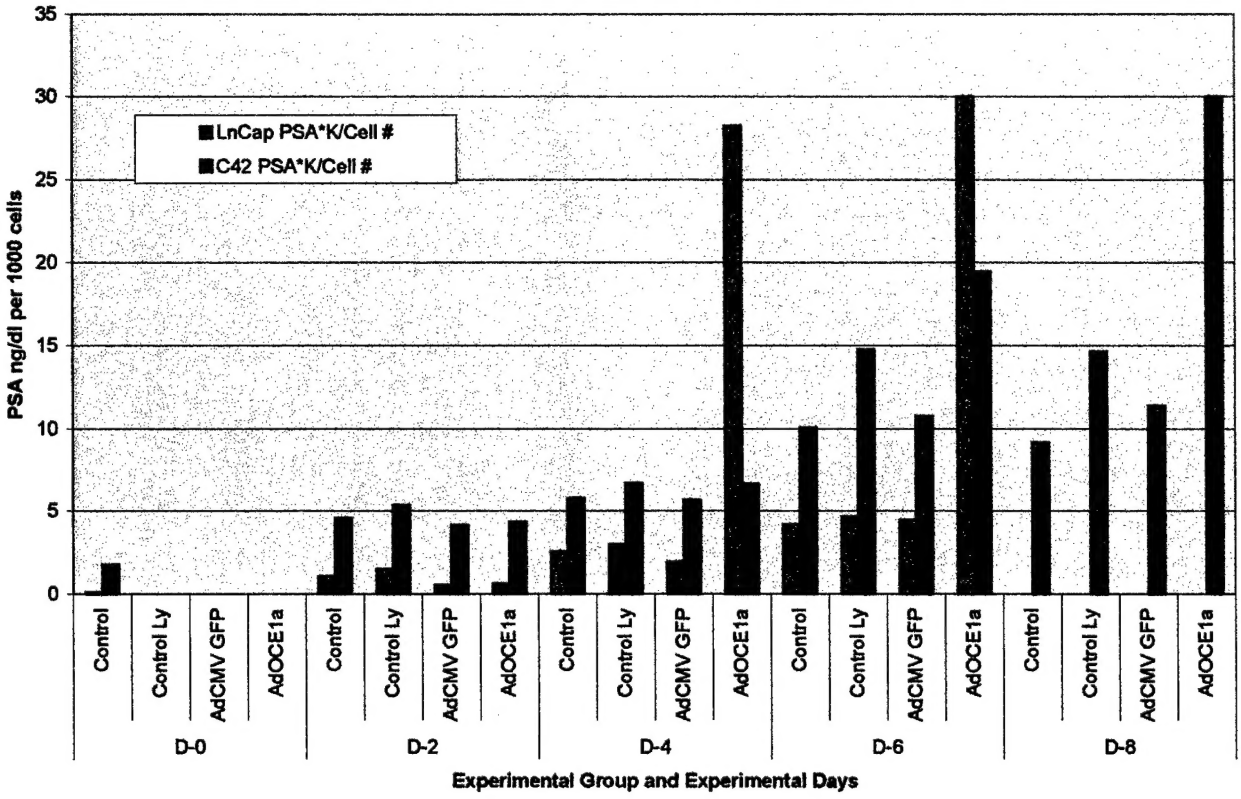


***Figure 6** illustrates the PSA production per 1000 human prostate cancer cells for clarity. In particular the PSA production in the media per cell does not change with the control GFP expressing reporter virus but does increase in within the Ad-OC-E1a group when viral mediated lysis is occurring. An in vivo time course study is proposed to address the PSA expression in the serum of animal with established tumors.*

Figure 6. PSA Expression and Adenoviral Infection



Task 5 is to analyze the results of the first 12 months to allow completion of this annual report. The biostatistician of the cancer center is reviewing the complete statistical analysis of this data presented in this annual report and complete statistical analysis will be included in the final report.

Task 6 is to conduct the microgravity experiments to assess the lytic ability of Ad-OC-E1a on various human cancer organiods. This task remains ongoing; therefore, the final results will be included in the final report. One unexpected occurrence was a malfunction of the microgravity chamber that required repair by the company and ultimately a new apparatus. I have been using these apparatus for the last 12 months without further malfunction. The initial analysis based on cellular morphology of the organoids was difficult *despite repeated experiments*. To facilitate the completion of the proposed experiments a series of GFP labeled and human prostate cancer cells have been established (figure 7). The intracellular markings will allow for more accurate assessment of the treatment effect.

Figure 7 demonstrates stable GFP expression of human prostate cancer cell line PC-3 (A), LNCaP (B) and C4-2 (C).

A. PC-3 MFG-EGFP-GALV **B. LNCaP MFG-EGFP-GALV** **C. C4-2 MFG-EGFP-GALV**

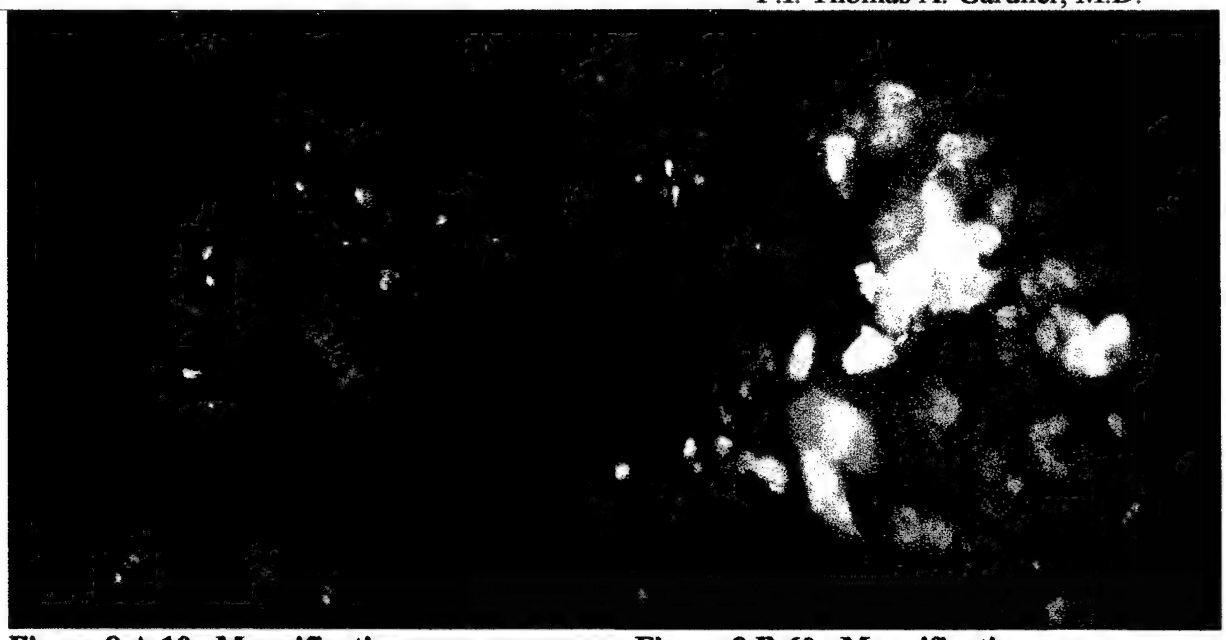


Figure 8 A 10x Magnification **Figure 8 B 60x Magnification**

Figure 8 *illustrates the ability of the GFP expressing cell lines to form xenographs similar to none GFP expressing cell lines. Figures 8 A (10x) and B (60 x) are intravital images by 3-D dual photon confocal microscopy with green cells corresponding to C4-2 GFP expressing cells and the red illustrating the vascular markings after infusion with Rhodamine Dextran (500,000 MW).*

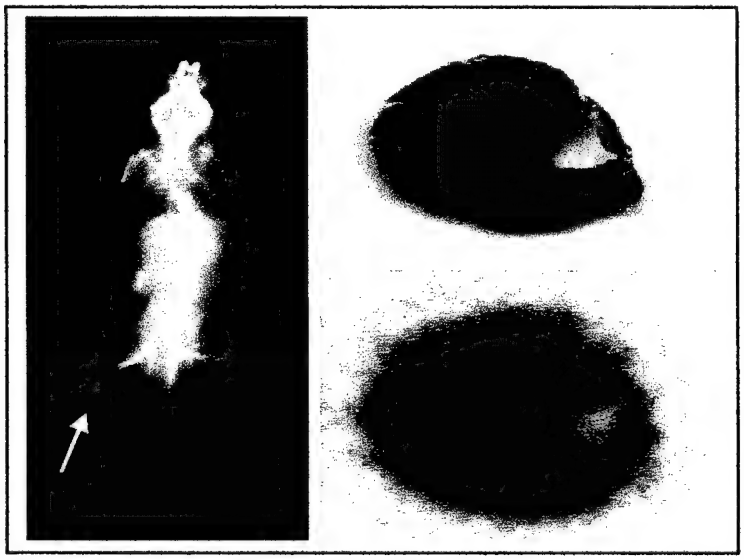


Figure 9. *illustrates microPET imaging of prostate tumor xenograft in nude mouse model. Using a nude mouse C4-2 interosseous model of human androgen-independent prostate cancer the potential of microPET imaging is demonstrated by figure 9. Figure 9(left panel) is a plain radiograph of a male nude mouse 8 weeks after intra-osseous injection of 1 million C4-2(GFP) cells into the tibia. The arrow points to the osteoblastic tibial lesion accounting for a serum PSA of 250 ng/dl, which can be contrasted to the contralateral tibia. Figure 9 (Right top panel). is a photomicrograph of the frozen section whole mount cross-section of the tibia at necropsy. Figure 9 (Right bottom panel) represents an autoradiograph of the same frozen cross-sectional slice after* ^{18}*F-FDG administration. The strong radioactive signal from this tissue attests to the biological activity of this androgen independent osteoblastic intraosseous human prostate cancer. Figures 8 and 9 demonstrate the maintained ability of the GFP expressing cells to form subcutaneous and intraosseous xenografts. Additionally the figures demonstrate novel techniques to evaluate actual tumor viability with the model systems being utilized. Although not described in the initial Statement of Work these establishment and validation of these non invasive techniques will provide more meaningful biological information of the model system and the treatment effect demonstrated by the Ad-OC-E1a Virus.*

Task 7 is to conduct subcutaneous xenograft experiments confirming the in vivo lytic activity post Ad-OC-E1a administration. This task remains ongoing, therefore, the results will be presented in the final report. Preliminary summary of the subcutaneous studies were published and are represented in figure 3 of appendix 1. This demonstrates the significant potential growth differential of PC-3 and LOVO tumors[14]. *MicroPET will be used to confirm tumor volume with viable tissue after exposure to the control and therapeutic virus.*

Task 8 is to evaluate the viral distribution time course and growth inhibition of intraosseous xenograft model. A portion of this task is completed, the methods, materials, results and discussion follow:

Methods and Materials: Intraosseous Xenografts with C4-2

Using an intraosseous model of androgen-independent prostate cancer, C4-2 cells were directly inoculated in the tibia or femur of nude mice and serum PSA was followed weekly until greater 5 ng/dl.[12]. When the Serum PSA was greater than 5 ng/dl these mice bearing intraossoeous xenografts were then treated with intralesional administration of Ad-OC-E1a of a control reporter virus Ad-CMV-B-Gal 1 x 10^9 pfu per lesion on one occasion. These animals were followed with weekly serum PSA's, radiographic findings and necropsy at 10 weeks post injection.

Results and Discussion Task 8

Figure 10 demonstrates the establishment of stable intraosseous xenografts using the C4-2 model. The y-axis is the serum PSA in ng/dl with each bar representing one mice on Day 0 of the intralesional study. The seven bars on the left received Ad-OC-E1a injections and the seven bars on the right received Ad-CMV-B-Gal injections. The starting PSA values were comparable in each group.

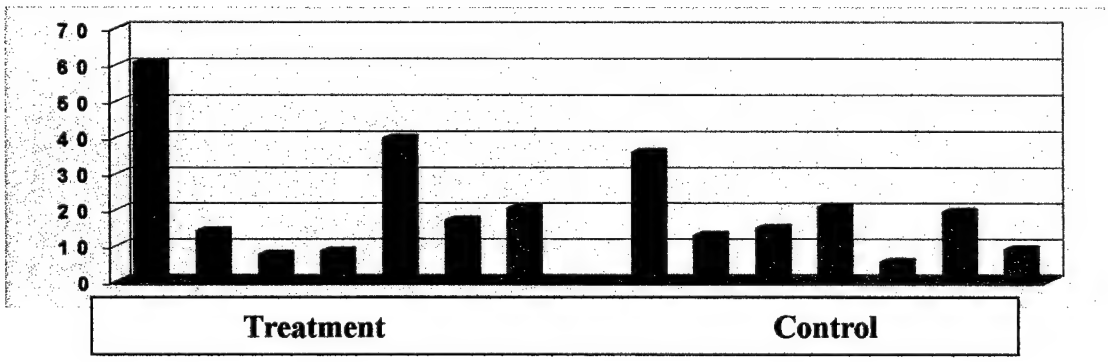


Figure 8 Day 0 Serum PSA (ng/dl) in Intraosseous C4-2 Model

Figure 11 and 12 show the weekly PSA readings for the control and treatment group, respectively. As predicted the control animals have increasing PSA values until sacrifice despite receiving Ad-CMV-B-Gal injections on day 0. Note that the scale of the y-axis on a 0-1000 scale while the treatment group is on a 0-100 scale to avoid dwarfing the treatment group's non-existent PSA values.

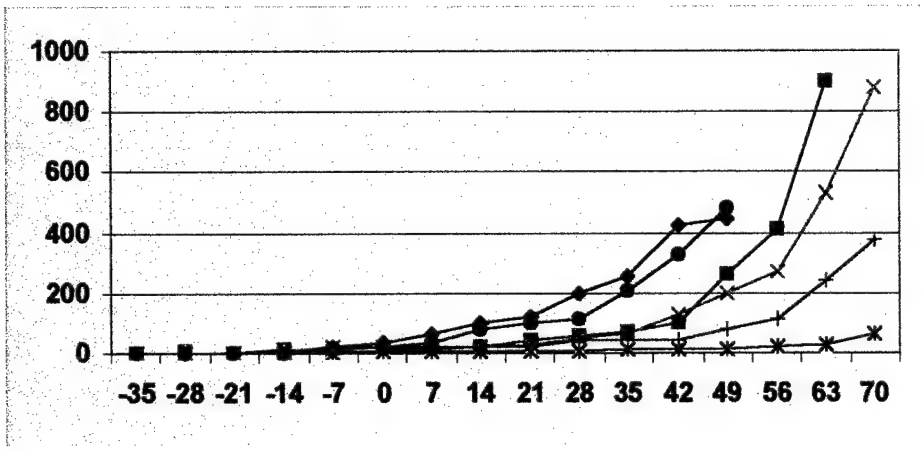


Figure 9: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-CMV-B-Gal (1 x 10^9 PFU) on Day 0.

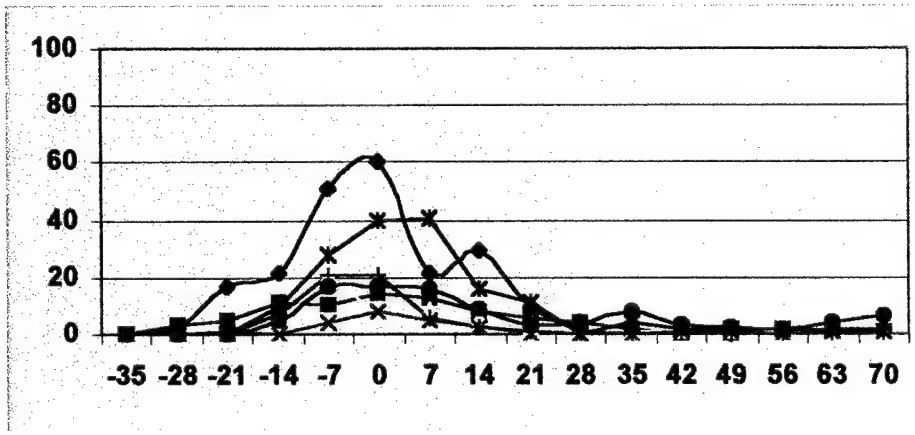


Figure 10: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-OC-E1a (1 x 10^9 PFU) on Day 0

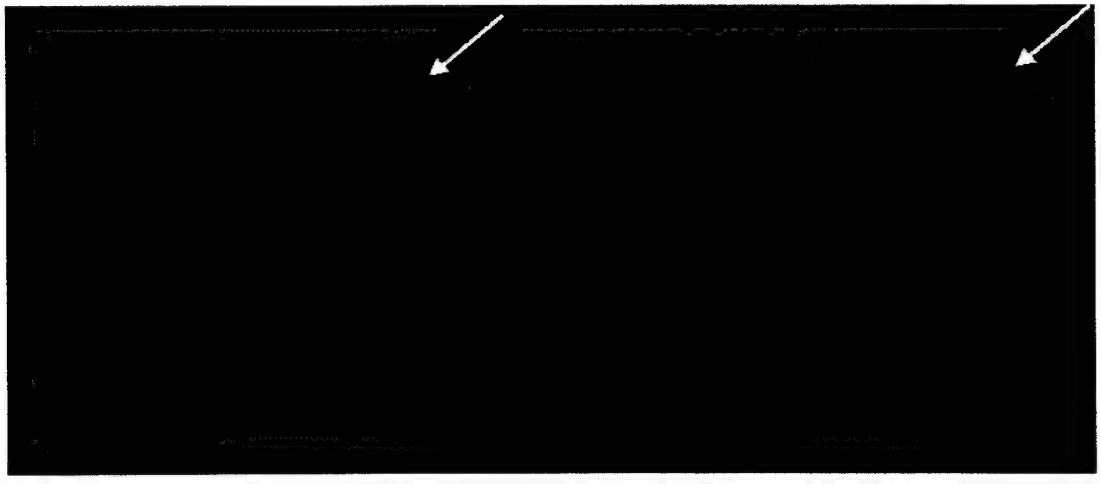


Figure 11 represents radiographs at sacrifice of one control (left) and one treated (right) mice, respectively. These radiographs illustrate the significant growth inhibition of intraosseous C4-2 tumors as evidenced by both PSA decline and normalization of radiograph after intralesional Ad-

OC-E1a. Note the large tumor on the tibia of the control animal and the normal radiographic picture of the mouse after receiving the injection of Ad-OC-E1a.

Task 9 is to examine the histopathological correlations with viral distribution study of task #8. This examination is ongoing and results will be reported in the final report.

Task 10 is to analysis the results prior to submission of the 2nd annual report. Final analysis of all results will be completed prior to the final report submission result.

Task 11 is to conduct the viral distribution after a variety of delivery techniques. A baseline viremia study of Ad-OC-E1a has been carried out and serum samples from a series of animals are being tested using quantitative PCR to discern the viral copy number in the blood and tissues. These preliminary studies will set-up the standards for the remainder of the viral distribution studies. *This viremia study was performed in immune intact rats to allow for serial blood samples to validate the PCR assay. The initial results revealed a second wave of viremia at day 14 which was unexpected due to the rodents inability to replicate the human adenovirus. The PCR analysis is being repeated to assure the reproducibility of this unexpected finding.* This task is ongoing.

Task 12 is the final data compilation, statistical analysis, manuscript preparation and final report preparation. To facilitate continued development of a clinical trial using Ad-OC-E1a some of the results were published Cancer Research as an "Advances in Brief". This delayed the more detailed manuscript to Molecular Therapy with the title being **"Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models."**

Key Research Accomplishments of Proposal

- **Confirmation of In Vitro specificity of OC promoter in OC+ cell lines.**
- **Confirmation of reproducibility of C4-2 intraosseous model.**
- ***Development of GFP marked PC-3, LNCaP and C4-2 human prostate cancer cell lines.***
- ***Confirmation of Intraosseous and Subcutaneous Xenografts formation of GFP-marked cell lines by MicroPET and Dual Photon Confocal Microscopy.***
- ***Confirmation of continued PSA production by human prostate cancer cells in culture after adenoviral infection.***
- **Confirmation of intraosseous tumor growth inhibition after intralesional injection of Ad-OC-E1a.**
- **Confirmation of intraosseous tumor growth inhibition after intravenous administration of Ad-OC-E1a.**
- **Initial demonstration of bimodal viremia of Ad-OC-E1a in immune intact rodents.**

Reportable Outcomes

- **Gardner TA**, Wada Y, Shirakawa T, Ko S-C, Kao C, Kim SJ, Yang L, Chung LWK. Osteocalcin promoter restricted adenoviral replication as a potential treatment of prostate cancer metastasis. Presented at the 8th International Conference on Gene Therapy of Cancer, San Diego, CA, December 1999. **1999 Vical Best Abstract Award**

- **Gardner TA, (PI)** OBA Gene Transfer Protocol #0010-426 "Phase I study of intratumoral injections of OCaP1(Ad-OC-E1a) for metastatic or locally recurrent prostate cancer, Part 1: Dose finding, Part 2: Index lesion escalation"

- *Matsubara, S., Wada, Y., **Gardner, T. A.,** Egawa, M., Park, M. S., Hsieh, C. L., Zhau, H. E., Kao, C., Kamidono, S., Gillenwater, J. Y. & Chung, L. W. (2001). A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. Cancer Res 61, 6012-9. (Appendix I). This was submitted as an Advance in Brief and final page proofs were submitted by Dr. Matsubara without additional grant support added.*

- ***Gardner TA**, Wada Y, Sukay M, Yang L, Brown L, Ko S-C, Cheng L, Chung LWK, Kao C. Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models. Molecular Therapy (in preparation submission held for additional PET Data).*

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Conclusions

This proposal is designed to test the hypothesis the adenoviral lytic replication cycle can be placed under the transcriptional regulation of the osteocalcin promoter. Since the initiation of the work the osteocalcin promoter continues to perform well in osteoblastic diseases such as osteosarcoma and prostate cancer in both pre-clinical and clinical settings[2; 14]. The work described above further illustrates the specificity of this promoter. The results to date can be simply divided into in vitro and in vivo results. In the in vitro assays demonstrate at a 100 fold better cell kill in the OC+ (LNCaP, C4-2, PC-3) and OC- (LOVO and PrSC). The completion of these in vitro experiments supported the early investigation in the in vivo setting. The in vivo finding of near-complete ablation of serum PSA in mice with established intraosseous C4-2 tumors compared to controls also provides strong evidence to support the current hypothesis under investigation.

The finding to data can be further supported by a recent clinical study from MD Anderson Cancer Center which demonstrated a true benefit to a therapy which targets the osteoblastic component of a prostate cancer mass[15]. This clinical trial performed by investigators from M. D. Anderson demonstrated improvements in median survival from 17 to 28 months in men with hormone-refractory prostate cancer receiving bone targeted therapy combined with a similar chemotherapy. This study combined with the findings to date would suggest that a Phase

I trial of Ad-OC-E1a may confirm the pre-clinical findings above and allow for the verification of the safety of Ad-OC-E1a as a novel therapeutic to target prostate cancer metastases. In summary, the proposal is being carried out close to the time line proposed with significant findings having already been discovered and published. These findings are heading to the clinic in the phase I trial of Ad-OC-E1a for men with metastatic prostate[16]. *The trial referred to in this annual report will be funded through independent funding mechanisms. In summary, I continue to make progress on the SOW but have expanded some aspects of the statement of work and have expanded on the outcome measures being utilized. At the completion of this proposal it is likely that the information generated will support grant application to the NCI and the National Gene Vector Laboratory to initiate a Phase I trial confirming the above preclinical findings.*

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Advances in Brief

A Conditional Replication-competent Adenoviral Vector, Ad-OC-E1a, to Cotarget Prostate Cancer and Bone Stroma in an Experimental Model of Androgen-independent Prostate Cancer Bone Metastasis[1]

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Abstract

Prostate cancer has a high propensity to metastasize to bone, which often resists hormone, radiation, and chemotherapies. Because of the reciprocal nature of the prostate cancer and bone stroma interaction, we designed a cotargeting strategy using a conditional replication-competent adenovirus to target the growth of tumor cells and their associated osteoblasts. The recombinant Ad-OC-E1a was constructed using a noncollagenous bone matrix protein osteocalcin (OC) promoter to drive the viral early *E1a* gene with restricted replication in cells that express OC transcriptional activity. Unlike Ad-PSE-E1a, Ad-OC-E1a was highly efficient in inhibiting the growth of PSA-producing (LNCaP, C4–2, and ARCaP) and nonproducing (PC-3 and DU145) human prostate cancer cell lines. This virus was also found to effectively inhibit the growth of human osteoblasts and human prostate stromal cells *in vitro*. Athymic mice bearing s.c. androgen receptor-negative and PSA-negative PC-3 xenografts responded to a single intratumoral administration of 2×10^9 plaque-forming unit(s) of Ad-OC-E1a. In SCID/bg mice, intraosseous growth of androgen receptor-positive and PSA-producing C4–2 xenografts responded markedly to i.v. administrations of a single dose of Ad-OC-E1a. One hundred percent of the treated mice responded to this systemic Ad-OC-E1a therapy with a decline of serum PSA to an undetectable level, and 80% of the mice with PSA rebound responded to the second dose of systemic Ad-OC-E1a. Forty percent of the mice were found to be cured by systemic Ad-OC-E1a without subsequent PSA rebound or tumor cells found in the skeleton. This cotargeting strategy shows a broader spectrum and appears to be more effective than systemic Ad-PSE-E1a in preclinical models of human prostate cancer skeletal metastasis.

Introduction

Genetic therapy for prostate cancer has been applied in preclinical animal models and in patients with localized and metastatic diseases (1–16). The prevailing approach is to target a single cell compartment such as the tumor epithelial or associated endothelial compartment (1, 2, 5–7). Examples of transgenes delivered to tumor cells include suicide genes (2–5), tumor suppressor (6, 8), antiangiogenesis (7), and immune modulators (9–13). Recently, Henderson *et al.* (1, 14, 15) demonstrated the efficacy of conditional replication-competent adenovirus with viral replication driven by a tissue-specific promoter, PSE,[3] for the treatment of prostate cancer. In this article, we present a novel strategy for cotargeting both tumor epithelial and bone stromal cells using a conditionally replicating adenovirus driven by a tissue-specific but tumor-restrictive promoter, OC (4, 16). This strategy was based on the well-established reciprocal cellular interaction that occurs between prostate cancer and prostate or bone stromal cells (17, 18). Evidence suggests that permanent phenotypic and genotypic alterations are induced in prostate cancer and bone stromal cells subsequent to tumor-stromal interaction (17, 19). The cotargeting strategy accomplishes maximal cell kill by eliminating not only the growth of tumor epithelium but also by interrupting the intercellular communication and reciprocal induction between prostate tumor and bone or prostate stromal cells (17–19).

OC, a noncollagenous Gla protein, was found to be produced exclusively by differentiated osteoblasts and is deposited onto bone matrices at the time of bone mineralization (4, 20, 21). The OC promoter contains several species-specific and overlapping regulatory elements (22–29). The "osteocalcin box" contains sites to bind factors such as homeobox MSX proteins, AP-1, AP-2, NF-1, viral core enhancer, c-AMP, vitamin-D, and glucocorticoid receptors (22–29). The osteoblast-specific *cis*-acting element OSE2 binds to the transcription activator of osteoblast differentiation, Osf2/Cbfa1 (28). Mouse OC promoter contains an additional OSE1 *cis*-acting DNA element (29) but has a nonfunctional vitamin D responsive element (22). The current study used mouse OC promoter to drive viral replication through the regulation of E1a, an adenoviral early gene required for viral replication (30). We described this cotargeting strategy by demonstrating: (1) Ad-OC-E1a is a highly efficient inhibitor of the growth of prostate cancer and bone and prostate stromal cells *in vitro*; (2) Ad-OC-E1a has a broad spectrum of cell kill activity that caused lysis in PSA-producing and -nonproducing prostate tumor, bone, and prostate stromal cells *in vitro*; (3) systemic administration of Ad-OC-E1a inhibited the growth of human prostate tumor established previously in the skeleton; and (4) the cotargeting strategy is superior to targeting a single cell compartment in which only the growth of prostate cancer cells is affected. Results of this study demonstrate for the first time that systemically administered Ad-OC-E1a induced regression of preexisting human prostate cancer growth in the skeleton irrespective of their prior PSA and AR status.

Materials and Methods

Cells and Cell Culture. LNCaP, an androgen-responsive, AR-positive, PSA-secreting human prostate cancer cell line, was derived from a cervical lymph node metastasis by Horoszewicz *et al.* (31). From this parental cell line,

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[3] The abbreviations used are: PSE, prostate-specific antigen enhancer; OC, osteocalcin; PSA, prostate-specific antigen; AR, androgen receptor; pfu, plaque-forming unit(s); FBS, fetal bovine serum; MOI, multiplicity of infection.

we derived a series of androgen-independent (defined as cells that are capable of forming PSA-secreting solid tumors when inoculated in castrated athymic male mice without the supporting stromal cells or extracellular matrices) and lineage-related LNCaP sublines (32, 33). One of the sublines, C4–2, remains AR- and PSA-positive and acquires osseous metastatic potential when inoculated either s.c. or orthotopically (32, 33). ARCaP is an androgen-repressed, low AR- and PSA-expressing human prostate cancer cell line established by our laboratory (34). This cell line is highly tumorigenic and metastatic and is a model to study advanced human prostate cancer (34). PC-3 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Kaighn *et al.* (35) from the bone marrow aspirates of a patient with confirmed metastatic disease. DU-145 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Stone *et al.* (36) from a patient with prostate cancer brain metastasis. Lovo, a colon cancer cell line, was established by Drewinko *et al.* (37) from a localized colon tumor tissue specimen and was kindly provided by Dr. L. Y. Yang, University of Texas M. D. Anderson Cancer Center, Houston, TX. WH, a cell line derived from a human bladder transitional cell carcinoma specimen, was established by Zhau *et al.* (38). 293 is a transformed human embryonic kidney cell line established by Graham *et al.* (39) with the cells expressing a complementing adenoviral E1 region that supports adenoviral replication. A human prostate fibroblast cell line, 9096F, was established by our laboratory from a surgical prostate biopsy specimen (40). A human bone stromal cell line, MG-63, was established from an osteosarcoma specimen and was obtained from the American Type Culture Collection (Rockville, MD). The PC-3, DU-145, and 293 cell lines were also obtained from American Type Culture Collection. In this study, C4–2 and 9096F cells were maintained in T medium (Life Technologies, Inc.) containing 10% FBS as described previously (32, 33). LNCaP, PC-3, DU-145, ARCaP, WH, and MG-63 cells were all maintained in T medium (Life Technologies, Inc.) containing 5% FBS. Lovo cells were maintained in F-12 Nutrient Mixture (Life Technologies, Inc.) containing 10% FBS. 293 cells were maintained in MEM (Life Technologies, Inc.) containing 10% FBS. The cells were fed three times per week with fresh growth medium and maintained at 37°C in 5% CO_2.

Construction and Production of the Replication-competent Ad-OC-E1a. All of the plasmids were constructed according to standard published protocols (41). Briefly, a *Bam*HI-XcaI fragment containing the backbone of an Ad5 vector from 549 bp to 5792 bp was digested from pXC 548C, a derivative of plasmid pXC1 (42), and inserted into pΔE1sp1B (obtained as a gift from Dr. Frank Graham, MacMaster University, Hamilton, Ontario, Canada) between the *Bam*HI and XcaI site to create a pΔBPAEII shuttle vector. A pOCE1a was constructed by inserting a 1370-bp fragment of murine OC promoter, which was cut from pII1.5 TK using *Xho*I and *Sal*I enzymes, into the *Xho*I site of pΔBPAEII to drive the Ad5 *E1a* gene. The shuttle pOCE1a vector was cotransfected with a replication-defective recombinant Ad vector, pJM17, into 293 cells by the *N*-[1-(2,3-dioleoyloxyl)propyl]-*N,N,N*-trimethylammonium-methyl sulfate (Boehringer Mannheim Biochemicals)-mediated transfection method (43) to generate a partially E3-deleted replication-competent adenovirus, Ad-OC-E1a. The resulting Ad-OC-E1a was demonstrated to replicate in a restricted manner only in cells that expressed OC promoter activity. The culture medium of the 293 cells showing complete cytopathic effect was collected and centrifuged at 1000 × *g* for 10 min. The pooled supernatants were aliquoted and stored at −80°C as primary viral stock. Viral stocks were propagated in 293 cells, and selected clones of Ad-OC-E1a virus were obtained by plaque purification according to the method of Graham and Prevec (44). One of the viral clones was selected, propagated in 293 cells, harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by subjecting the cells to centrifugation, and the virus in the cell lysate was purified by CsCl gradient centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. The viral titer was determined by plaque assay as described previously (2, 8, 45). Other control viruses used in this study, Ad-CMV-pA and Ad-CMV-β-gal, were constructed, plaque purified, and propagated in 293 cells using a similar procedure. The specificity of Ad-OC-E1a replication in cells was assessed by determining the titer of the virus after infecting 293 (7.7 ± 3.2 pfu/cell), C4–2 (15 ± 8 pfu/cell), and WH (0.047 ± 0.021 pfu/cell) cells ($n = 3$).

Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Deparaffinized primary human prostate cancer specimens and lymph node and bone metastatic specimens were obtained from the Department of Urology and Pathology, University of Virginia School of Medicine, Charlottesville, VA and McGill University, Montreal, Quebec, Canada. Tissues were treated with 3% H_2O_2, blocked with SuperBlock (Scytek Laboratories, Logan, UT), and reacted with a monoclonal IgG OC antibody (5–16 μg/ml; OC 4–30 antibody purchased from Takara Shuzo, Otsu, Japan). The antibody staining signals were amplified by a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories, San Ramen, CA). Background and negative control staining were routinely obtained by the use of a purified control mouse IgG and a clinical human colon cancer specimen, respectively. OC stain was visualized after reacting the conjugated peroxidase with either an AEC Chromogen, 3-amino-9-ethylcarbazole, or a diaminobenzidine as described previously (2, 8, 34, 38, 45). Positive OC is defined by ≥15% of the cell populations reacted positively with the OC antibody.

***In Vitro* Cell Growth Assay.** LNCaP, C4–2, PC-3, DU-145, ARCaP, 293, WH, Lovo, MG-63 or 9096F cells (5×10^3) were plated in 24-well plates. After 24 h, the cells were infected with Ad-OC-E1a with a range of concentrations from 0.01 to 5 MOI (or pfu/cell, which was estimated to be 0.2–100 virus particles/cell) for 2 h. Cells infected with Ad-CMV-pA or Ad-CMV-β-gal served as negative controls. Cell numbers were measured 3 days later by the crystal violet assay using an automated E max spectrophotometric plate reader (Molecular Devices Corp., Sunnyvale, CA) as described previously (2, 8, 45).

Assessment of Adenoviral Infectivity in Mouse and Human Bones. To determine whether normal mouse or healthy human bones are susceptible to Ad infection, we performed two studies. An Ad-CMV-β-gal (1×10^9 pfu) was injected into the femur of an adult mouse, and the bone was harvested 3 days later for histochemical analysis of β-gal activity using a method established previously (2, 8, 44). Additionally, a normal bone specimen harvested from a 69-year-old man with bone fracture was cultured in T medium containing 0.6% soft agar. A human prostate cancer PC-3 xenograft cultured similarly in 0.6% soft agar was also infected and secured as a control. The tissue specimens were exposed to Ad-CMV-β-gal (1×10^9 pfu) and were processed 3 days after infection. After harvesting bone and prostate tumor specimens, tissues were first washed in PBS and fixed in 0.05% glutaraldehyde at 4°C for 24 h. Bone specimens were put in PBS for 24 h after fixing and decalcified with 0.25 M EDTA in PBS (pH 7.4) at 4°C for 5 days. After decalcification, the specimens were stained overnight in a solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Prostate tumor specimens were processed as described previously and were stained similarly as described above for β-galactosidase activity (2, 8, 44).

***In Vivo* Animal Experiment.** To demonstrate oncolytic activity and tumor specificity of Ad-OC-E1a, athymic mice (20–25 g) were inoculated s.c. with 1×10^6 PC-3 or Lovo cells suspended in 100 μl T medium containing 5% FBS. When the tumor became palpable (4–5 mm in diameter), the animals were randomly assigned to two experimental groups: group 1, Ad-OC-E1a; and group 2, Ad-CMV-β-gal. A single dose of virus (2×10^9 pfu) was injected intratumorally in mice. After administration of the test viruses, tumor sizes were measured and recorded.

To evaluate the effect of systemic Ad-OC-E1a in the intraosseous prostate tumor model, 1×10^6 C4–2 cells were injected into the bone marrow space of the right tibial bone in castrated male SCID/bg mice according to procedures published previously (46). Blood specimens (~100 μl) were obtained from the tail vein for PSA assay once per week. Serum PSA was determined by microparticle ELISA using an Abbott IMx machine (Abbott Laboratories, Abbott Park, IL). After the detection of serum PSA elevation, a single dose of 25 μl Ad-OC-E1a, 2×10^9 pfu (or 4×10^{10} virus particles)/animal, was administered i.v. to mice. When serum PSA rebound had occurred, animals were treated with the second or third i.v. injection of the same dose of the test virus at the specified time points as indicated. Serum PSA was monitored weekly, and histopathology and X-ray of the tumors were routinely assessed when the animals were sacrificed.

Results

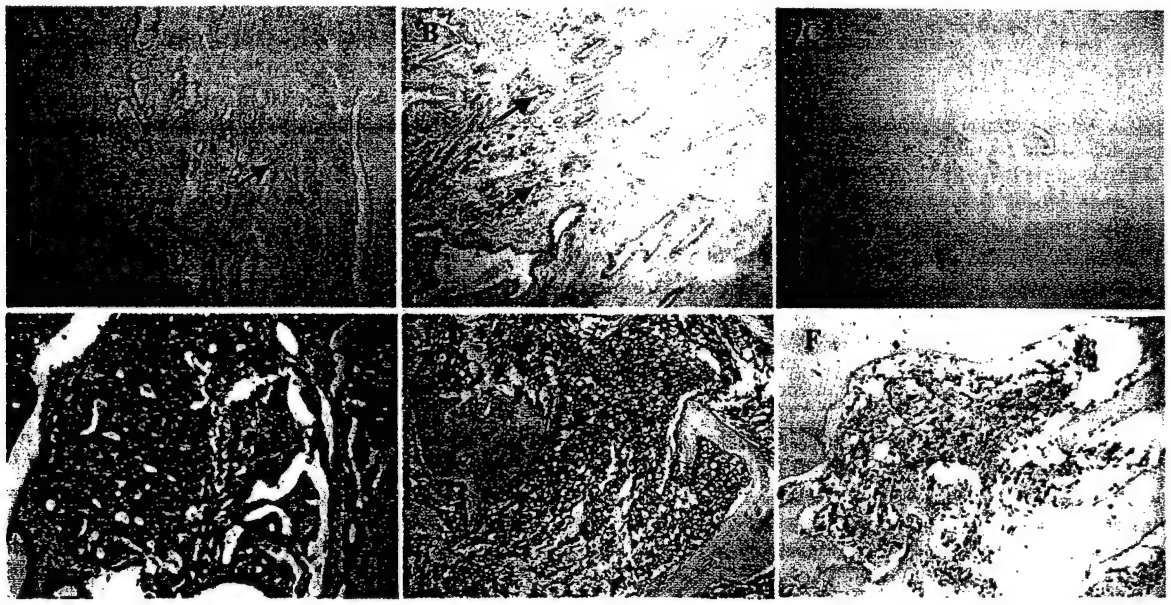
Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Whereas OC has been shown to be a specific marker expressed exclusively in osteoblast-lineage cells, OC was also found to be expressed in calcified normal smooth muscle tissues, vascular endothelial pericytes, and benign tumors

(47–50). We demonstrated OC expression (see *arrows*) by immunohistochemistry in a primary prostate tumor stroma (Fig. 1*A*), primary prostate tumor epithelium and stroma (Fig. 1*B*), a prostate tumor lymph node (Fig. 1*D*), and a bone (Fig. 1*E*) metastatic specimen. Positive OC stain was found in 85% (23/27) of the primary prostate tumor specimens and in 100% of the prostate tumor lymph node (12/12) and bone (10/10) metastatic specimens. OC stains are generally less intense in the primary prostate cancer but are very intense in metastatic prostate cancer (lymph node and bone) specimens. Background immunohistochemical staining of OC was demonstrated by the use of a control mouse IgG in a primary human prostate tumor (Fig. 1*C*), a human colon cancer (data not shown), and human prostate tumor bone metastatic specimens (Fig. 1*F*). Positive immunostaining of OC was also demonstrated in normal human bone specimens (data not shown) and tumor-associated osteoblasts (Fig. 1*E*).

Cytotoxicity of Ad-OC-E1a to Prostate Cancer Cell Lines *in Vitro*: Independent of Endogenous PSA and AR Status. To assess the cytotoxicity of Ad-OC-E1a, we exposed a number of human prostate cancer cell lines, LNCaP, C4–2, PC-3, DU-145, and ARCaP, *in vitro* to a wide range (0.01–5 MOI) of Ad-OC-E1a vector. We used 293, or WH and Lovo cells as positive or negative controls, respectively. We observed that whereas exposure of LNCaP and C4–2 cells to 5 MOI of Ad-OC-E1a inhibited the growth of these cells by 70% (Fig. 2*a*), this same dose of Ad-OC-E1a was ineffective in blocking the growth of WH and Lovo cells, which exhibit barely detectable or nondetectable OC promoter activity (data not shown). Cells infected similarly by the control viruses, either without an insert (Ad-CMV-pA) or with β-gal insert (Ad-CMV-β-gal), were also unaffected even when exposed to 5 MOI of the virus (Fig. 2*a*). Next, we evaluated the efficacy of Ad-OC-E1a in several other human prostate cancer cell lines that either expressed a very low level (*e.g.*, ARCaP) or nondetectable (*e.g.*, PC-3 and DU-145) level of PSA and AR. Fig. 2*b* shows that all of the tested human prostate cancer cell lines were sensitive to Ad-OC-E1a-induced cell lysis *in vitro* irrespective of their intrinsic levels of PSA and AR expression. In addition, we also evaluated the effects of Ad-OC-E1a on the growth of a human prostate fibroblast and a human osteosarcoma cell line *in vitro*. As demonstrated in Fig. 2*c*, Ad-OC-E1a infection induced significant cell lysis in both cultured human prostate fibroblast (*e.g.*, 9096F) and osteoblast (MG-63) cell lines.

Abolishing s.c. PC-3 Tumor Growth *in Vivo* with Intratumoral Ad-OC-E1a. To establish the specificity of Ad-OC-E1a in inhibiting prostate tumor growth *in vivo*, we compared the activity of this virus on the growth of s.c. human prostate PC-3 tumors established previously with that of human colon Lovo tumors (serve as a negative control) *in vivo*. Fig. 3 shows that Ad-OC-E1a effectively inhibited the growth of PC-3 but not Lovo tumors when injected intratumorally. PC-3 tumors injected similarly with Ad-CMV-β-gal (data not shown) and Lovo tumors with Ad-OC-E1a exhibited a slight inhibitory effect on tumor volumes. These data are consistent with the observation that OC promoter activity is present in PC-3 but not in Lovo cells and that Ad-OC-E1a induced marked cytotoxicity in PC-3 but not Lovo cells *in vitro* (Fig. 2, *a* and *b*).

Systemic Ad-OC-E1a Eliminated C4–2 Human Prostate Tumors Established Previously in the Skeleton. A PSA-secreting and androgen-independent human LNCaP prostate cancer subline, C4–2, was chosen to evaluate the efficacy of systemic Ad-OC-E1a. Prostate tumors established previously in the skeleton with increased



Prostate Cancer Specimens	Positive/Total (%)
Primary Prostate Cancer	23/27 (85%)
Lymph Node Metastasis	12/12 (100%)
Bone Metastasis	10/10 (100%)

Fig. 1. Immunohistochemical demonstration of the presence of OC in primary and metastatic human prostate cancer specimens. Positive OC stain (*arrows*) was detected in primary cancer-associated stroma (*A*) and both prostate stroma and tumor epithelium (*B*). Positive immunostaining of OC was also found in lymph node (*D*) and bone (*E*) metastasis. Background immunostaining was found in control primary (*C*) and bone metastatic (*F*) prostate cancer when these specimens were stained by mouse IgG. Accumulated incidences of OC-positive specimens (positive/total) are expressed on *bottom panel*.

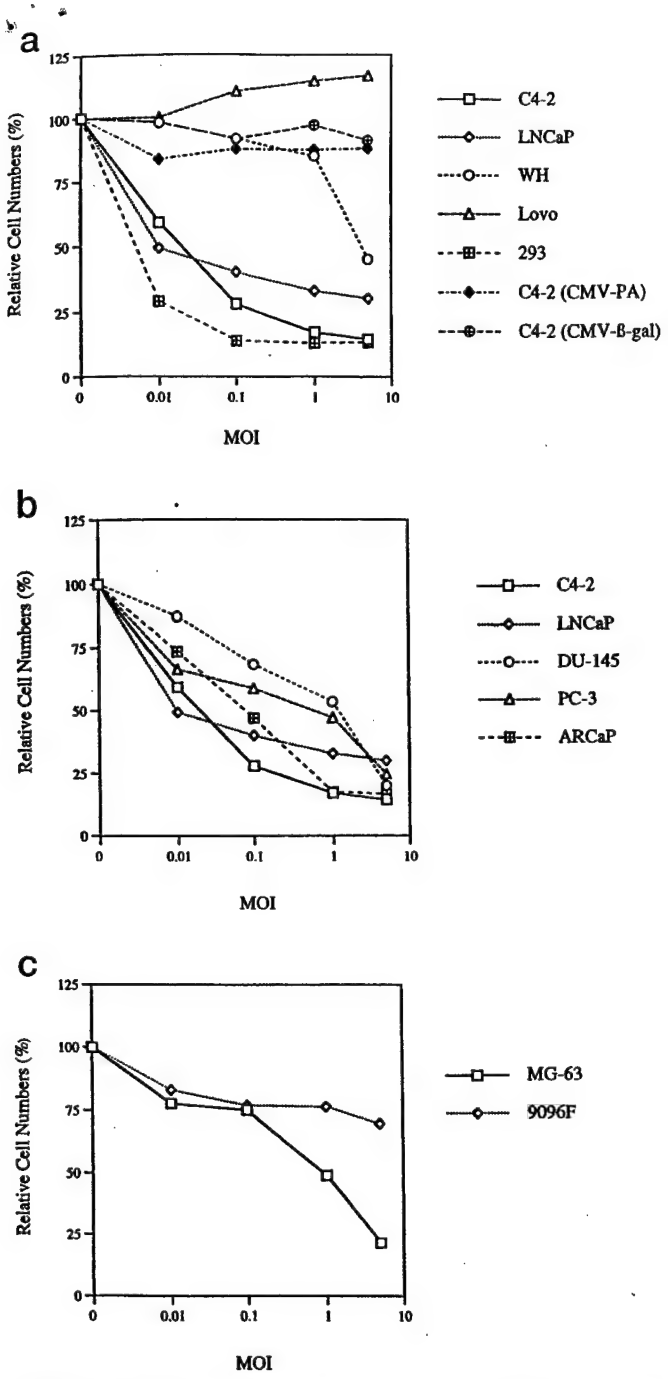


Fig. 2. Inhibition of human prostate cancer and bone and prostate stromal cell growth *in vitro* by the replication-competent Ad-OC-E1a. Cell growth was assessed *in vitro* in the presence of Ad-OC-E1a, Ad-CMV-β-gal, or Ad-CMV-pA. The percentage of cell viability was measured on day 3 after infection of the test virus (range, 0.01–5 MOI or pfu/cell). Results showed that although Ad-CMV-pA and Ad-CMV-β-gal did not affect the growth of C4–2, Ad-OC-E1a inhibited cell growth of C4–2 and 293 in a viral concentration-dependent manner. Ad-OC-E1a was not effective in inhibiting cell growth of WH and Lovo cells, because these cells lack OC promoter activity and OC expression. Results also showed the comparative aspect of efficacy of Ad-OC-E1a-induced cell lysis in C4–2, LNCaP, PC-3, ARCaP, and DU-145 human prostate cancer cell lines. Note PSA- and AR-positive (LNCaP, C4–2), PSA-negative (PC-3, DU-145), and marginally PSA- and AR-positive (ARCaP) human prostate cancer cell lines are all susceptible to Ad-OC-E1a-induced cell lysis. Additionally, results showed both human prostate stromal cells and osteoblasts are also sensitive to Ad-OC-E1a-induced cell lysis. Data represent the average of triplicate experiments determined with SD within 15% of the mean.

serum PSA were subjected to systemic Ad-OC-E1a administration. Serum PSA was followed weekly, and upon PSA rebound, Ad-OC-E1a treatment was repeated on animals. Six animals were evaluated in this study. Fig. 4*a* shows that in one control untreated mouse, serum PSA underwent marked elevation from a basal 10 ng/ml at 6 weeks to 630 ng/ml at 15 weeks (*Panel A*). This profile of rapid PSA rise is consistent with our previous reports (2, 33, 46). Serum PSA profiles of Ad-OC-E1a-treated mice are shown in Fig. 4*a* from *Panel B* to *F*. Several variants of the PSA responses were noted. Mice 2 and 3 responded to systemic Ad-OC-E1a treatment with a complete regression of the skeletal tumors (Fig. 4, *b* and *c*), and a PSA nadir (*i.e.*, nondetectable PSA) was achieved for >15 weeks (see *Panels B* and *C* of Fig. 4*a*). These two mice are considered as cured by systemic Ad-OC-E1a treatment, because there was no subsequence PSA rebound, and no tumor was detected in the skeleton. Mice 4 and 5 responded to systemic Ad-OC-E1a with a marked and rapid PSA decline. PSA nadir in these mice was maintained for a variable period ranging from 1 to 6 weeks (see *Panels D* and *E* of Fig. 4*a*). These mice appear to have variable rebound of PSA during subsequent observation. Mouse 6 responded favorably to systemic Ad-OC-E1a initially with a PSA nadir lasting for 5 weeks. However, this mouse gradually escaped from systemic Ad-OC-E1a growth inhibition and appeared less responsive to the second and third dose of Ad-OC-E1a treatment (Fig. 4*a*, *Panel F*).

Gross Morphology, Histopathology, and Immunohistochemistry of Prostate Tumor Xenografts Harvested from the Skeleton in Mice Treated with Systemic Ad-OC-E1a. Marked gross anatomical differences were found between the control mice and mice responsive to Ad-OC-E1a (Fig. 4*b*). As shown by X-ray and gross anatomy, systemic Ad-OC-E1a induced marked regression of prostate tumors in

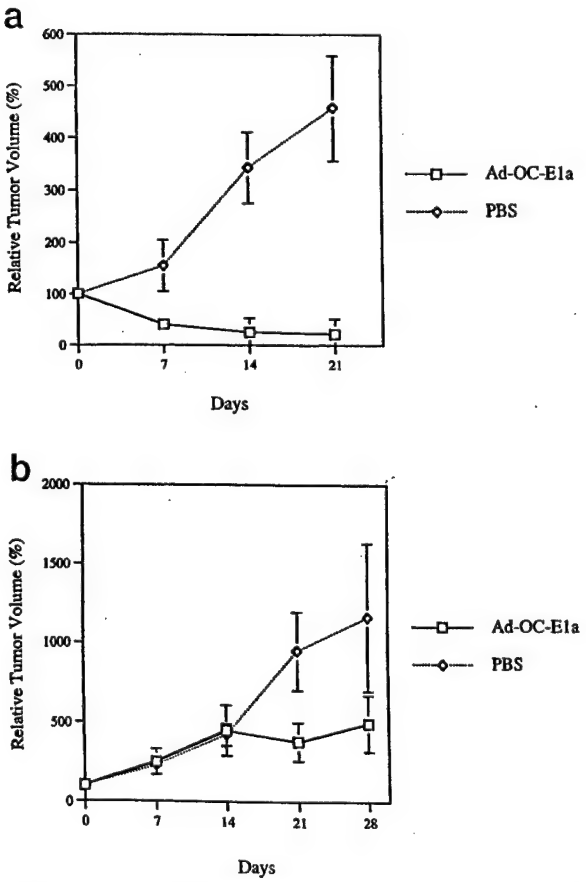
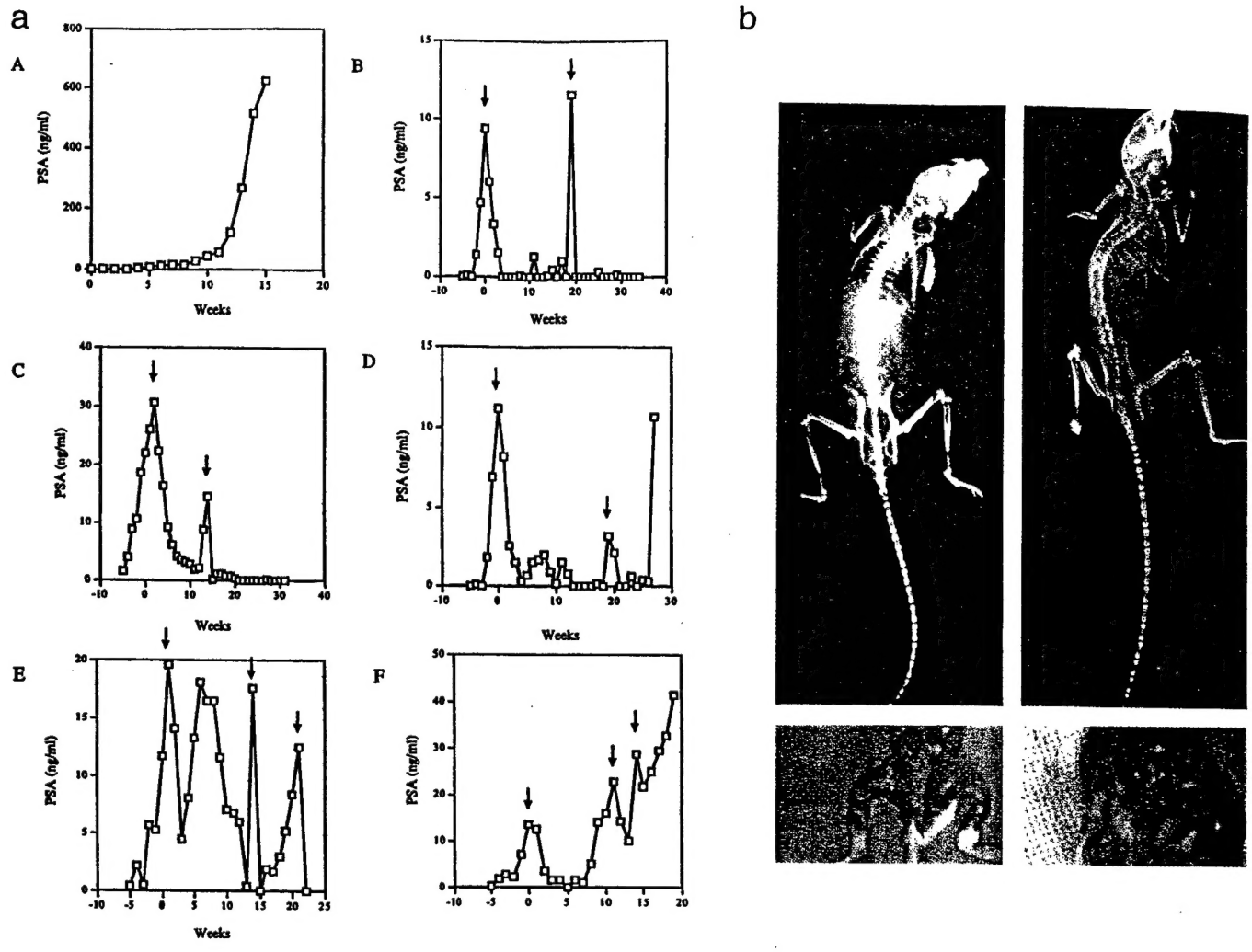
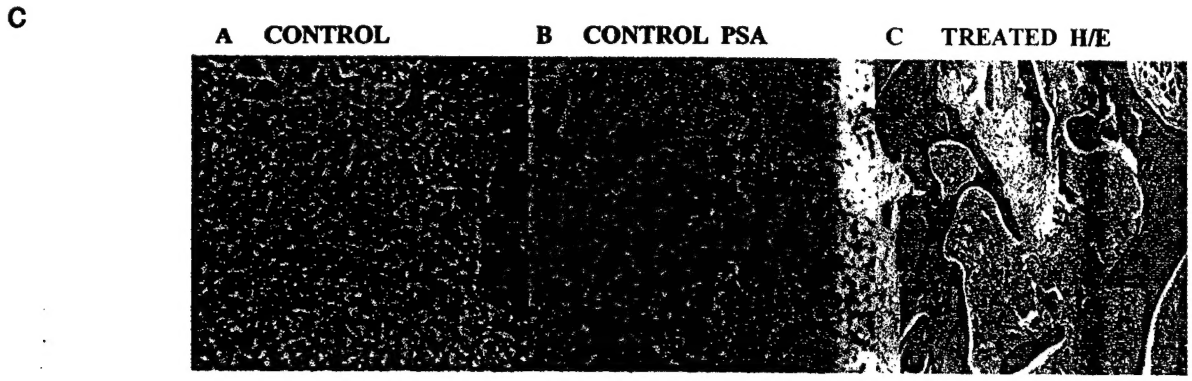


Fig. 3. Treatment of tumor xenografts with recombinant adenoviruses. Tumor xenografts were grown s.c. in athymic nude mice. Tumors were treated with recombinant viruses and PBS by intratumoral injection on day 0 and measured weekly. The data represent mean; *bars*, ± SD. *a*, PC-3 xenograft tumors were treated with Ad-OC-E1a and PBS (n = 4). *b*, Lovo xenograft tumors were also treated with Ad-OC-E1a and PBS (n = 4).





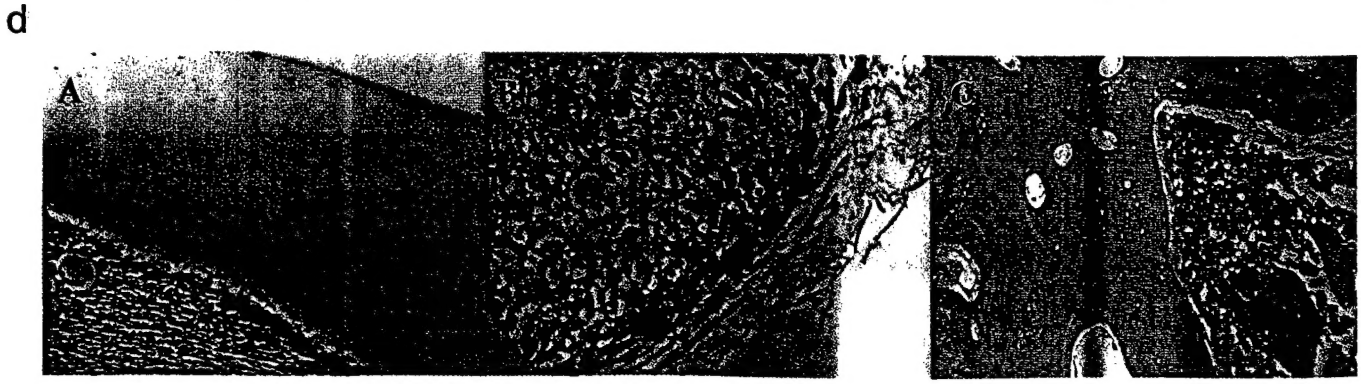


Fig. 4. *a*, demonstration of i.v. Ad-OC-E1a on serum PSA levels in SCID/bg mice injected intraosseously with C4-2 cells. *Panel A*, serum PSA level of an untreated control mouse after intraosseous injection of 1×10^6 C4-2 cells. Note exponential rise of serum PSA in the untreated mouse (mouse 1). *Panels B–F*, mouse 2–6 serum PSA levels of animals injected

the tibia. This marked improvement was confirmed by examining the histopathological section of the tumors obtained from the control and Ad-OC-E1a treated animals. Fig. 4*c* shows that in comparison to the systemic Ad-OC-E1a, the untreated mice have large tumors and strained positively by PSA antibody in the skeletal specimens (Fig. 4*c, Panels A* and *B*), whereas the prostate tumors cured by systemic Ad-OC-E1a failed to yield positive histopathology in the skeleton (Fig. 4*c, Panel C*) or positive immunohistochemical staining of PSA in the representative specimens (data not shown). We also compared the adenoviral infectivity in mouse bone *in situ*, human prostate PC-3 xenografts, and human bone maintained *in vitro* in soft agar. Results of this study showed that a single intraosseous administration of Ad-CMV-β-gal infected effectively the mouse bone cells without affecting the cortical bone (Fig. 4*d, Panel A*). *In vitro* Ad-CMV-β-gal efficiently infected upper layers of PC-3 tumor cells (Fig. 4*d, Panel B*) but not human bone cells (Fig. 4*d, Panel C*) maintained as explants in soft agar.

Discussion

Cancer therapies using adenoviral vectors can be divided into two broad categories, replication-defective and replication-competent (51). Because of the difficulties in infecting all of the cancer cells with adenoviral vectors, numerous laboratories have designed various versions of viral constructs with the primary goal of increasing the efficiency of viral infectivity (or transgene expression) or viral replication in competent tumor cells without damaging the normal tissues. One such approach relies on the ability of "bystander" genes such as hsv-TK or cytosine deaminase incorporated into replication-defective adenoviral vectors to convert prodrugs into biologically active growth-inhibitory products that elicit efficient cell kill even in cells that were not transduced with virus-bearing genes (2–5, 52, 53). The construction of replication-competent ONYX-015 lacking E1b, a MR 55,000 protein, can conceptually replicate in tumor cells that lack functional p53 protein (54). Conditional activation of viral gene expression and replication have also been achieved using tissue-specific promoters such as PSA or PSE for prostate cancer (1, 14, 15), α-fetal protein for liver cancer (55), and tyrosinase for melanoma (56, 57). Modification of adenoviral gene structure by introducing adenoviral death protein has achieved higher efficiency of viral replication (58). In the present study, we explored the possibility of using a novel tissue-specific (*i.e.*, osteoblast-specific) and tumor-restrictive (*i.e.*, restricted to calcified benign and malignant tumors) OC promoter to drive adenovirus replication in cells that contain OC promoter activity. This version of adenoviral vector allows the virus to replicate in both tumor epithelium (Fig. 2, *a* and *b*) and its supporting stromal cells including a human prostate stromal cell line (Fig. 2*c*) and a human osteoblast cell line (Fig. 2*c*). Thus, Ad-OC-E1a could potentially inflict maximal cell kill through, primarily, viral replication in tumor epithelium, and secondarily, by destruction of intercellular communication between tumor and stroma, inducing cell lysis in prostate fibromuscular stromal cells, osteoblasts, and potentially vascular endothelial pericytes (47–49). In experimental coculture studies both *in vitro* and *in vivo*, induction of osteoblast cell death by hsv-TK/acyclovir or ganciclovir also markedly inhibited the growth of prostate tumor cells.[4]

Because OC expression is highly restricted to maturing osteoblasts (20–29, 47), Ad-OC-E1a may potentially damage bone and alter the balance between rate of bone resorption and formation. This concern has been addressed, and our study is summarized below. First, the cortical bone of both mouse and human restricts adenoviral infection. We observed that whereas mouse bone marrow is highly susceptible to adenoviral infection, human bone marrow including maturing osteoblasts appeared to be more resistant to adenoviral infection (Fig. 4*d*). Therefore, it is possible that in humans, Ad-OC-E1a replication may be limited to proliferating and maturing osteoblasts, which express OC promoter activity. Second, intraosseous administration of Ad-OC-hsvTK plus i.p. ganciclovir in intact adult mice did not induce any abnormal histopathology of the skeleton (4, 59). In fact, OC has been shown as an inhibitor of bone mineralization by preventing the growth of mineral crystals in an *in vitro* assay (60). This role of OC is consistent with the transgenic OC knockout mouse model where the destruction of OC-expressing cells by hsv-TK resulted in increased bone mass and bone formation (61, 62).

The tissue-specific and tumor-restrictive OC promoter potentially has several advantages over other prostate-specific promoters such as PSA or PSE enhancer (1, 15), human kallikrein 2 (hK2; 14), or prostate-specific membrane antigen (63). One advantage is that OC is expressed prevalently by human primary and metastatic prostate cancers with expression found in both tumor epithelium and/or surrounding stromal compartment (see Fig. 1). Another advantage is that OC expression is not limited to prostate tumors and was also found expressed by other calcified benign and malignant tissues such as smooth muscle plaques associated with heart valve and blood vessels (48, 49); benign tumors (50); and malignant osteosarcoma, brain, thyroid, breast, lung, and ovarian tumors (unpublished results) irrespective of their basal PSA and AR status. This is significant because it was estimated that ~20% of prostate cancer patients do not have elevated PSA despite the detection and progression of the disease (64). In addition, although AR gene amplification and overexpression were detected in almost 30% of the clinical prostate cancer specimens (65), AR-mutant or AR-null prostate cancer cells and tissues were nevertheless commonly observed (64–67). On the basis of the above observations, it is possible that PSA and/or AR-negative tumors may be responsive to Ad-OC-E1a but not to Ad-PSA-E1a-induced cell lysis.

Several previous publications demonstrated that an Ad vector-mediated toxic gene, hsv-TK, expression driven by OC promoter, inhibited the growth of osteosarcoma (16, 45, 68) and its metastasis (69) and inhibited prostate tumor growth both *in vitro* and *in vivo* (4, 16). Although intratumoral administration of Ad-OC-TK was used in most of these earlier studies, we observed significant remission of osteosarcoma lung metastasis and improvement of survival by systemic administration of Ad-OC-TK (69). The ability of systemic Ad-OC-TK to exert antitumor effects on osteosarcoma pulmonary

[4] C-L. Hsieh, *et al.*, unpublished observation.

with Ad-OC-E1a (*arrows*, 2×10^9 pfu of Ad vector was administered via tail vein after detecting a rising serum PSA in animals that received intraosseous injection of 1×10^6 C4-2 cells). Systemic Ad-OC-E1a administration induced rapid decline of serum PSA, and 4/5 (80%) animals responded markedly to repeated Ad-OC-E1a treatment. *b*, gross morphology and X-ray evidence indicating the capability of Ad-OC-E1a in eradicating the growth of intraosseous human prostate tumor xenografts. Administration of Ad-OC-E1a via tail vein induced marked tumor regression in the treated mouse (*left panels*). *c*, regression of prostate tumors by Ad-OC-E1a is supported by histopathological evidence of the absence of prostate tumor cells in the skeleton (*Panel C*). In the untreated animals, both tumor cells (*Panel A*) and PSA (*Panel B*) were observed in the skeletal specimen. *d*, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of normal mouse and human bone infected with Ad-CMV-β-gal (1×10^9 pfu). Note whereas the mouse bone marrow cells are susceptible to Ad-CMV-β-gal infection, the cortical bone of the mouse is resistant to Ad infection (*Panel A*). *Panel B*, human PC-3 prostate tumor is susceptible to Ad-CMV-β-gal infection when maintained as explants on soft agar. Normal human bone exposed to this virus appears to exhibit background β-gal activity suggesting that normal human bone cells may be resistant to Ad vector infection (*Panel C*).

metastasis without causing liver toxicity (59, 69) indicated the importance of considering the selection of tumor- or tissue-specific promoters to drive the expression of therapeutic genes or viral replications for cancer therapy. In this context, it is clear that conditional replication-competent adenovirus may have the advantage of amplifying the input of oncolytic virus and help the spread of agents to adjacent cells in a highly promoter- and cell-dependent manner. (1, 5, 14, 15, 51, 54, 58). In this study, we demonstrated substantial efficacy of systemic Ad-OC-E1a for the treatment of androgen-independent prostate cancer skeletal xenografts. However, we demonstrated that to eliminate the preexisting human prostate tumor xenografts in the bone, Ad-OC-E1a administration must be repeated. We obtained evidence that all of the mice responded initially to Ad-OC-E1a therapy (as judged by serum PSA response) and only one mouse (20%) escaped Ad-OC-E1a effects gradually and became an Ad-OC-E1a nonresponder. Forty percent (2/5) of the Ad-OC-E1a-treated mice have undergone complete tumor regression and are considered "cured" in the present protocol. Reasons why mice may lose their response to Ad-OC-E1a are presently unclear, but it is reasonable to suggest that Ad-OC-E1a infectivity may be reduced in the resistant tumors through a decreased coxsackie adenoviral receptor on tumor cell surface or a rapid clearance of Ad vectors at tumor sites from systemic circulation. Whereas the current protocol may be applicable to the treatment of clinical prostate cancer skeletal metastasis, some precautions need to be observed: (*a*) Ad-OC-E1a replication in normal human tissues requires more extensive testing, and human bone and human prostate cancer chimeric xenografts grown s.c. may be ideal for this evaluation (70); and (*b*) serum PSA response may be an indication but not the proof of tumor regression (71). Even if there is a potential pitfall in using altered serum PSA as the indicator for an antitumor effect, it is firmly established that serum PSA response does correlate with improved survival, pain relief, increased hemoglobin level, normalization of bone-derived alkaline phosphatase, weight gain, or improved performance status of prostate cancer patients (72). Smith *et al.* (73) found that a decrease in the serum PSA level of $\geq$50% at 8 weeks was correlated with significantly increased survival. Such data validate the use of changes in the serum PSA level as a response parameter in trials of therapy in prostate cancer. In this study, we have shown that the PSA response correlated well with the histopathologies of prostate tumors in the skeleton and demonstrated the efficacy of systemic OC promoter-driven conditional replication-competent adenovirus in abolishing the growth of preexisting prostate tumors in bone.

In summary, we have established a novel replication-competent adenoviral therapy using a tissue-specific and tumor-restrictive OC promoter to drive the replication of adenovirus for the treatment of prostate cancer metastasis in an experimental human prostate cancer skeletal xenograft model. Ad-OC-E1a was shown to be effective in eliminating preexisting androgen-independent prostate tumors in the bone without adverse effects on mouse bone. This study establishes for the first time that cotargeting prostate cancer and bone stroma may be an effective strategy for destroying human prostate tumor skeletal metastasis.

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